Functional diversity of CMV-specific T-cells is maintained in older people and significantly associated with protein specificity and response size

Martha Bajwa[1,*], Serena Vita[2,*], Rosanna Vescovini[3], Martin Larsen[4,5], Paolo Sansoni[3], Nadia Terrazzini[6], Stefano Caserta[1], David Thomas[1], Kevin A. Davies[1], Helen Smith[6], and Florian Kern[1]

[1]Division of Medicine, Brighton and Sussex Medical School, Brighton, United Kingdom

[2]Institute Pasteur, Cenci-Bolognetti Foundation, Department of Public Health and Infectious Diseases, University Sapienza of Rome, Rome, Italy

[3]Dipartimento di Medicina Interna e Scienze Biomediche, Università di Parma, Parma, Italy

[4]Inserm UMR-S1135, Centre d'Immunologie et des Maladies Infectieuses (CIMI-Paris), Paris, France

[5]AP-HP, Groupement Hospitalier Pitié-Salpêtrière, Département d'Immunologie, Paris, France

[6]School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, United Kingdom

[7]Division of Primary Care and Public Health, Brighton and Sussex Medical School, Brighton, United Kingdom

[*]both authors should be considered first authors

Footnotes

Conflict of interest statement:

MB: No COI; SV: No COI; RV: No COI; ML: ML is inventor of the Polyfunctionality Index (patent number: WO2013127904) and proprietary owner of the Funky Cells ToolBox software.; PS: No COI; NT: No COI; SC: No COI; DT: No COI; KD: No COI; HS: No COI; FK: FK is a named owner/inventor on a patent describing protein-spanning peptide pools for T-cell stimulation (EP1257290 B1). Reagents covered by this patent were used in this study.

Funding statement

This work was supported by The Dunhill Medical Trust, UK (Grant R278/0213)

Meeting presentations

None

Corresponding Author Information:

Prof. Florian Kern

Medical Research Building

Brighton & Sussex Medical School

University of Sussex

Brighton BN1 9PS

Tel: +44 (0) 1273 877876

E-mail: f.kern @bsms.ac.uk

Abstract

Background: Parallel up-regulation of several T-cell effector functions ('polyfunctionality') is believed to be critical for the protection against viruses but thought to decrease in large T-cell expansions, in particular at older ages. The factors determining T-cell polyfunctionality are incompletely understood. Here we revisit the question of CMV-specific T-cell polyfunctionality, including a wide range of T-cell target proteins, response sizes, and participant ages. **Methods:** Polychromatic flow-cytometry was used to analyze the functional diversity (CD107, CD154, IL-2, TNF, IFN-γ) of CD4 and CD8 T-cell responses to 19 CMV proteins in a large group of young and older UK participants. A group of oldest old people (>85years) was included to explore these parameters in exceptional 'survivors'. Polyfunctionality was assessed for each protein-specific response subset by subset and in aggregate across all proteins using the novel polyfunctionality index (PI).

Results: Polyfunctionality was not reduced in healthy older compared to young people. However, it was significantly related to target protein specificity. For each protein it increased with response size. In the oldest old overall T-cell polyfunctionality was significantly lower.

Discussion: Our results give a new perspective on T-cell polyfunctionality and raise the question if maintaining polyfunctionality of CMV-specific T-cells at older ages is necessarily beneficial. **(abstract word count: 200 words)**

Introduction

The numbers of fresh, naïve cells leaving the thymus declines early in life. However, T-cell numbers are maintained at a constant level in adults [1] because memory T-cells proliferate to compensate for this shortfall. Cytomegalovirus (CMV) appears to be a uniquely effective driver of this compensatory proliferation, sometimes producing very large T-cell expansions [2, 3]. It is unclear how these changes affect the functional diversity ('polyfunctionality') of T-cells. Polyfunctionality is an important T-cell quality, which has been linked to protection in certain vaccine models [4-7]. Several studies have explored T-cell polyfunctionality at older ages but it is not known whether this varies with respect to CMV protein target and/or response size. Previous work on CMV-related T-cell polyfunctionality has focused on just one or two frequently recognized, 'dominant' CMV proteins [8, 9] but it is unlikely that the polyfunctionality of one or two dominant responses sufficiently captures the overall polyfunctionality of the T-cell response to this pathogen (including dominant and subdominant responses) [10]. We therefore decided to revisit this interesting question taking into account a wider, more representative range of CMV target proteins than previously studied. By analyzing 5 functional read-outs for CD4 and CD8 T-cell responses to the 19 most representative CMV target proteins [11] in young and older people, we explored whether polyfunctionality is fully preserved in older people and how this is linked to target protein specificity and response size.

Methods

Ethics statement

This study was approved by the UK National Research Ethics Service (NRES) (09/H1102/84). Written informed consent was obtained from all participants. The study was conducted in agreement with the Declaration of Helsinki.

Blood donors

As part of a larger study, CMV-specific T-cell responsiveness was examined in CMV-infected (IgG-seropositive, 'CMV+') 'young' (19-35 years old) and 'older' (60-85 years old) individuals in East Sussex, UK. Healthy young volunteers included university students/staff; healthy older volunteers were recruited through general practitioners affiliated to the UK National Institute of Health Research (NIHR) Primary Care Research Network. Exclusion criteria were; known immunodeficiency (including HIV-infection), organ transplantation, use of immunosuppressive or immunomodulating drugs within the last year (excluding acetylsalicylic acid ≤ 100mg/day), current neoplastic disease or treatment for cancer within the previous 5 years, insulin dependent diabetes, moderate or advanced renal failure, liver disease, endocrine disorders (except corrected thyroid dysfunction), autoimmune disease, dementia/mental incapacity, alcohol/other drug abuse, acute infection or illness in the last 4 weeks, and raised body temperature (>37.5° C).

An additional 22 CMV+ individuals with previously known T-cell responsiveness to CMV were recruited in Parma/Italy as a comparison group of particularly advanced age (85 – 102 years) (referred to as 'oldest old'). Inclusion criteria for Italian volunteers were;

minimum age 85 years, known CMV responsiveness; exclusion criteria were; evidence of endocrine (except thyroid dysfunction), autoimmune and neoplastic diseases, acute infections or illness in the last 2 months, renal or liver failure, and use of immune-modulatory medications (including steroids, non-steroidal anti-inflammatory agents, acetylsalicylic acid >100mg/day, or immunosuppressive drugs). Individuals with cerebrovascular and/or cardiovascular disease were accepted, as the goal was to include individuals representative of such an advanced age cohort. Demographics are shown in **Table 1**.

CMV status

CMV Serology (Architect CMV IgG, Abbot, Maidenhead, UK) was performed in the Brighton and Sussex University Hospital Trust (BSUHT) virology laboratory.

Peripheral blood mononuclear cell (PBMC) Isolation and activation

PBMC were isolated from fresh, sodium heparin-anticoagulated venous blood by density gradient centrifugation (Ficoll-Hypaque, PLUS Healthcare, Buckinghamshire, UK) [12]. PBMC were resuspended at 5 x 10^6 cells/mL in complete RPMI (Fisher Scientific, Loughborough, UK) containing 10% fetal calf serum (Fisher). Twenty-five µg per peptide of CMV peptide-pools ("PepMix", JPT Peptide Technologies, Berlin, Germany) was dissolved in 100 µL of dimethyl-sulfoxide (Sigma-Aldrich, Gillingham, UK). 2 µL of peptide solution, 1.5 µL of anti CD107a (BD) and 0.5 µL of Monensin (BD) were added to 56 µL of complete media and placed in 4.5 mL polystyrene tubes (BD). Then 200 µL of PBMC suspension was added and tubes were incubated at 37°C in a

standard incubator in a humidified 5% CO_2 atmosphere. Brefeldin A (5 µg/ml; Sigma) was added after 2 hours and samples were incubated for additional 14 hours. Final concentrations of peptide were 1 µg/mL per peptide for each pool. Staphylococcus enteroxin B (SEB) (Sigma) was dissolved in DMSO and used at 1 µg/ml (final concentration) as positive stimulation control; peptide solvent (DMSO) alone served as a negative control. At the end of the incubation time 100 µl of a 20 mM EDTA buffer was added to each tube and incubation continued for 10 min at 37°C. EDTA buffer was freshly prepared by adding Na-EDTA (Sigma) to 'wash buffer' consisting of PBS supplemented with 0.5% bovine serum albumin and 0.1% sodium azide (Sigma). Tubes were vortexed and then incubated for a further 10 min at 37°C. After spinning at 400g for 8 min at 4°C, pellets were washed once more with wash buffer (same centrifuge settings) and then resuspended for staining.

CMV Peptide Pools

Peptides (15 amino acids length, 11 overlaps between adjacent peptides) covering the amino acid sequences for CMV proteins UL28, UL32, UL36, UL48, UL55, UL82, UL83, UL86, UL94, UL99, UL103, UL122, UL123, UL151, UL153, US3, US24, US29, US32 were prepared by solid-phase synthesis [11, 13]. All peptides were quality controlled by mass spectroscopy and HPLC. Peptide purity was > 80%. One single pool was generated for each protein, except for the largest protein, UL48, for which two pools were generated ('Pepmix', JPT Peptide Technologies, Berlin, Germany). Freeze-dried pools were stored at -80°C. The 19 original CMV peptide pools were arranged in 16 stimulation pools, of which 12 pools contained just one protein (expected to elicit

frequent responses) and 4 pools contained 2 proteins each (expected to elicit less frequent responses) (**Table 2**).

Antibodies

We used the following fluorescence-conjugated monoclonal antibodies and staining reagents: anti-CD3-v500, anti-CD8-Allophyocyanine(APC)-H7, anti-CD27-Phycoerythrine(PE) , IL-2-Fluoresceine-iso-thio-cyanate(FITC) , TNF-α- Alexa 700 , CD107a-APC (all BD Biosciences, Oxford, UK), anti-CD4-Peridinin chlorophyll (PerCP), anti-IFN-γ PE-Cy7 (Cyanine 7), anti-CD154 Pacific-Blue (BioLegend, Cambridge, UK), anti-CD45RA-ECD (Beckman Coulter, UK), Yellow live-dead stain (Invitrogen, Paisley, UK).

Intracellular Cytokine Staining

Staining antibodies were added and tubes incubated for 30 minutes at 4°C. After a further wash, red blood cells were lysed with FACS Lysing solution (BD) and then permeabilized with BD Permeabilizing solution 2 (BD) according to the manufacturer's instructions. Cells were then stained intracellularly following the same steps as used for surface staining. Following a final wash, pellets were resuspended and fixed in PBS containing 0.5% paraformaldehyde (Sigma) prior to acquisition on an LSRII flow cytometer using FACSdiva 6.1 software (BD).

Data analysis and Gating strategy

After stimulation activated cells were enumerated by flow-cytometry using five simultaneous T-cell activation read-outs, CD107, CD154, IL-2, TNF, IFN-γ. Data analysis was performed with FlowJo-v9.x software (TreeStar Inc., Ashland, USA). Details of the gating strategy are available on-line (**Supplementary Fig. S1**). Individual gates were set on activation marker-positive CD4 and CD8 T-cells. These were combined using the Boolean gate function in FlowJo generating 32 subsets (31 functional subsets, 1 without any of the tested functions). Net subset frequencies were determined by background subtraction (subset by subset). Responses were considered negative if they were not identifiable by at least one activation marker and a visible cell cluster exceeding 1/10,000 CD4 or CD8 T-cells (0.01%).

Polyfunctionality

SPICE software was used to visualize and analyze non-overlapping functional subsets [14]. The polyfunctionality index (PI) algorithm was obtained from 'FunkyCells ToolBox' version 0.1.0 beta (www.FunkyCells.com) [15]. To calculate the PI, each subset defined by a given number of displayed functions has a weight assigned which is then multiplied with the subset frequency. The PI is the sum of these products (PI = $\sum_{i=0}^{n} F_i \cdot \left(\frac{i}{n}\right)^q$ where *Fi* is the frequency of cells performing *i* simultaneous functions, *q* is the polyfunctionality parameter determining the weight of the subsets, *n* is the number of possible functions). The polyfunctionality parameter *q* was set to 1 as previously described [15]. Samples containing less than 0.1% activated events were not included in correlations of PI and other parameters.

Statistical analysis

SPSS v22 software (IBM, London, UK) was used for statistical analysis. Non-parametric tests (Mann-Whitney) were used to compare groups unless normality of data distribution was assumed (Q/Q plots, and the Kolmogorov-Smirnov test). Differences between more than two groups (independent samples) were tested with the Kruskal-Wallis test. Differences with respect to several parameters (related samples) were tested with Friedman's ANOVA. Where appropriate T-cell frequencies were log-transformed to normalize distribution or improve visual presentation. P-values <0.05 were considered significant for single endpoints. Where there were multiple end-points Bonferroni correction was applied to adjust the significance level from $p \leq 0.05$ to $p \leq 0.05/n$, where n is the number of endpoints.

Results

The functional T-cell subset distribution is very similar in young and older UK participants

CMV-specific CD4 and CD8 T-cells were analyzed in a healthy UK cohort (two age groups) and a cohort of oldest old Italians. The main interest in the latter was to contrast findings in the older group (within the normal life expectancy) against exceptional survivors. They were not considered examples of 'normal' ageing. After stimulating PBMC with 19 representative CMV target proteins the five simultaneously measured T-cell activation read-outs (CD107, CD154, IL-2, TNF, IFN-γ) gave rise to n=32 functional (Boolean) subsets when positive/negative populations were

gated for each marker ($n=2^5$), however, the subset negative for all markers was discounted from the analysis leaving 31; these were visualized using SPICE software [14]. However, none of the functional CD4 or CD8 T-cell subsets was significantly different between the young and the older UK participants when analyzing UL83-specific responses (**Supplementary Fig. S2**); this was also true for UL55-specific CD4 and UL123-specific CD8 T-cells (not shown). For this type of analysis the significance threshold was set at $p \leq 0.0016$ (Bonferroni multiple endpoint correction, 31 end-points).

In order to reduce the complexity of the information we determined the proportions of T-cell subsets with specific numbers of parallel functions (1 to 5) (**Fig. 1A**). These were very similar in young and older people, except for a slightly (but significantly) higher proportion of UL83-specific CD4 T-cells with two functions observed in the older people. We also studied the total percentage of cells expressing each activation marker in order to explore functional marker dominance (note that such subsets overlap). However, no significant differences were visible (**Fig. 1B**). Nevertheless, the data shown in Fig. 1 and supplementary Fig. 2 suggests that many smaller subset differences might exist and the lack of statistical significance could be due to the small group sizes particularly among the young. The number of participants with a UL55-specific CD4 and UL123-specific CD8 T-cell responses were only 6/26 and 9/26 among the young, but these were the second and third most frequently recognized proteins in the study. This meant it was highly unlikely that we would be able to recruit sufficient numbers of responders for each protein in order to complete a representative comparison between the age groups *protein by protein*. We therefore decided to base the comparison of polyfunctionality on

all present T-cell responses in the two age groups, irrespective of the recognized target protein. In addition, we decided to use an algorithm that would assign an aggregate measure of polyfunctionality to each response, because interpreting group differences with respect to multiple percentages of subsets with slightly different functional characteristics appeared to be too complex. We, therefore, applied the recently introduced polyfunctionality index (PI) to our data. The PI is a compound measure, which elegantly reduces the complexity inherent in multiple subset comparisons to a single index number [15, 16].

The PI is very similar in the young and older UK groups but lower in the oldest old 'survivors'

We first determined the PI for every single response and then the average PI for CD4 and CD8 T-cell responses in each individual. This allowed us to include *all* responses to *all* 19 proteins in our comparison. Surprisingly, the average PI appeared to be slightly higher in the older participants, but this difference was not statistically significant (**Fig. 2A**).

However, the oldest old, who were considered examples of exceptionally successful ageing, showed a significantly lower PI in their CD4 and CD8 T-cell responses than the older participants (**Fig. 2B**). When comparing the UL83-specific T-cell response between the older and the oldest old in more detail using SPICE [14], larger percentages of functionally focused subsets (one or two functions) present in the latter were consistent with this observation (**Supplementary Fig. S3**). In particular, they had higher frequencies of UL83-specific CD4 T-cells up-regulating CD154 and IFN-γ or

degranulation alone. Among UL83-specific CD8 T-cells the subsets displaying IFN-γ alone or IFN-γ and degranulation were significantly increased compared to the older group. The subset displaying degranulation alone also seemed markedly increased but this difference did not achieve statistical significance. Concerning UL55-specific CD4 T-cell responses, the oldest old had higher frequencies of the subset displaying only degranulation (mirroring the UL-83 specific response) but there were no differences in the other subsets (data not shown). For UL123-specific CD8 T-cells, by contrast, no significant differences were found (data not shown).

Polyfunctionality varies between responses targeting different proteins but is related to response size

We also applied the PI to the comparison of protein-specific responses among the UK cohort (young and older together). For this purpose we selected the 5 most frequently recognized CD4 and CD8 T-cell target proteins. Whilst differences for CD4 T-cells were apparent but not statistically significant (**Fig 2C, left**), significant differences were observed for CD8 T-cells (**Fig. 2C, right**). In order to elicit how individual functional subsets reflected these differences for the most frequently recognized proteins we used SPICE [14]. Visualization of all individual subsets suggested that UL55-specific CD4 T-cells included significantly larger subsets displaying IFN-γ and TNF, or IFN-γ, TNF and degranulation (CD107) than UL83-specific CD4 T-cells. Meanwhile, UL123-specific CD8 T-cells included significantly larger subsets displaying IFN-γ and TNF in combination or alone than UL83-specific CD8 T-cells **(Supplementary Fig. S4**). An increase of subsets focused on single effector markers is consistent with a loss of polyfunctionality.

Interestingly, there seemed to be different 'levels' of polyfunctionality for responses targeting different proteins (Fig. 3 B). Of note, these levels *per se* did not seem to depend on whether average responses to a protein were large or small. For example, UL123 induced significantly larger CD8 T-cell responses than UL83 (**Fig. 2C, bottom right**), but the responses to UL83 were significantly more polyfunctional (**Fig. 2C, top right**). A similar trend was seen for CD4 T-cell responses to UL55 (bigger) and UL83 (more polyfunctional) but differences were not significant. However, with respect to individual proteins, there was a significant association between PI and response size. This was observed for CD4 T-cell responses to UL83 and UL55 (**Fig. 2D, top**) as well as for CD8 T-cell responses to UL83 and UL123 (**Fig. 2D, bottom**). Similar but statistically non-significant associations between response size and PI were observed for one additional CD8 and two additional CD4 protein targets among the top 5 recognized proteins in each T-cell compartment (the numbers of cases were lower than for the three dominant proteins mentioned above).

Finally, the differences in PI between UL83 and UL55-specific CD4 T-cells as well as UL83 and UL123-specific CD8 T-cells ($p=0.018$ and $p=0.066$, respectively, data not shown) were preserved in the oldest old, albeit not statistically significant in CD8 T-cells. An association between response size and PI was visible in the oldest old for the same proteins as in the UK cohort but only as a non-significant trend (due to a lack of sufficient numbers of observations, data not shown).

Discussion

The present study provides a comprehensive analysis of the polyfunctionality of CMV-specific T-cells at different ages. It covers CD4 and CD8 T-cell responses to 19 different CMV target proteins, examines healthy participants in two different age groups, and also includes a cohort of successfully aged, oldest old, for comparison. We analyzed five simultaneous functional readouts and applied the recently introduced PI in addition to conventional subset-by-subset analysis. Use of the PI enabled us to analyze (aggregate) polyfunctionality in individuals and groups of individuals, responses to different proteins, as well as correlations between polyfunctionality and response size. Our results agree with published reports indicating that age *per se* is not linked to reduced polyfunctionality of CD8 T-cell responses to a range of different organisms [17] or altered CD4 and CD8 T-cell responses to superantigens [18]. For statistical robustness, when comparing the PI between T-cell responses of different protein specificities we focused on the most frequently recognized ones (U83, UL123, UL55). But when comparing the PI of CMV-specific T-cell responses between age groups, all responses to all proteins were accounted for. Since for each individual, the average PI across their specific CD4 and CD8 T-cell responses was computed prior to comparing these averages between the age groups, our approach provided unprecedented coverage of CMV target proteins for the analysis of polyfunctionality. It convincingly demonstrates that ageing within the expected lifespan in the UK (and many other countries in the world) has no obvious detrimental effect on the functional diversity of CMV-specific T-cells.

With respect to what determines T-cell polyfunctionality, it is interesting that responses to different target proteins had different PIs. This might be explained by the presentation of different CMV proteins by APCs in different contexts [19] and/or the efficiency of antigen presentation, including T-cell receptor antigen sensitivity and the amount of antigen available [20]. It is conceivable that the same protein might elicit responses of different polyfunctionality (and magnitude) in cohorts of different genetic background, i.e. certain HLA-types might contribute to the presentation of peptides that are recognized with higher TCR affinity and avidity than others [21]. These aspects clearly warrant further study.

In addition, a striking and significant association between the PI and CD4 and/or CD8 T-cell response size was observed for responses to UL83, UL123, and UL55 in the young and older donors. It remains unclear whether this holds generally true for sub-dominant proteins. In the oldest old we saw the same trend that polyfunctionality increased with response size but there were not enough cases for correlations to reach statistical significance.

The present study significantly extends recent work in a large group of (exclusively) older people with respect to UL83-specific T-cell responses. That study showed a correlation between the increase of certain polyfunctional subsets (for example exhibiting TNF, IFN-γ, Perforin expression as well as degranulation) and the size of the UL83-specific T-cell response. However, unlike in the present study, T-cell polyfunctionality was not assessed by an aggregate measure such as the PI and other proteins were not included. Also, a comparison between older and oldest old was not made.

Perhaps unexpectedly, results in the oldest old demonstrated decreased polyfunctionality, with an overall reduced PI of both CD4 and CD8 T-cell responses. In agreement with this, our detailed subset analysis indicated that several subsets with just one or two of the measured functions were increased (e.g. CD8 T-cells displaying IFN-γ alone, or IFN-γ and degranulation). Studies conducted over a decade ago suggested that in very old people the expansion of UL83-specific T-cells (recognizing specific epitopes in a narrow HLA-context) was linked to functional exhaustion [22, 23]. The individuals examined in that work were similar in age to our oldest old group and the decrease in polyfunctionality we have measured in this group might be a correlate of the 'functional exhaustion' reported in these earlier studies, which used fewer activation markers.

In any case, it seems to go against the notion that higher polyfunctionality is associated with better protection if it is reduced in the oldest old [15]. Protection from CMV-disease (probably the most important role of CMV-specific T-cells) might well be provided by a very small number of CMV-specific cells [10], but decreased polyfunctionality of large responses in very old people might reduce CMV-associated immune pathology. Such may include for example vascular pathology contributing to cardiovascular events and stroke, which are major causes of death in older people [12, 24-27]. It is important to remember in this context, that cells with greater polyfunctionality also produce larger and potentially more harmful quantities of effector cytokines [9]. A link between CMV-specific T-cell polyfunctionality and increased immune pathology would undermine the idea that T-cell polyfunctionality at older ages is necessarily a good thing. It might be of advantage for long-term survival, by contrast, if the level of pathogen control is balanced

against the risk of 'collateral' tissue damage. Maybe this is achieved to an extent by reduced polyfunctionality of CMV-specific T-cell responses in very old people. This is an important question to be answered in light of the growing interest in the possible role of CMV in a range of age-associated pathologies [28-30].

In conclusion, our study has revealed new aspects of CMV-specific T-cell polyfunctionality, in particular the previously unknown effects of target protein specificity and response size. While we now have a better understanding of this complex issue, the discovery that T-cell polyfunctionality in the oldest old long term 'survivors' is actually reduced has raised the question if increased T-cell polyfunctionality in older people could potentially limit survival.

Acknowledgements

The National Institutes of Health Research (NIHR) kindly assisted us with participant recruitment through the Primary Care Research Network (PCRN).

References

1. Gruver AL, Hudson LL, Sempowski GD. Immunosenescence of ageing. J Pathol **2007**; 211:144-56.

2. Pawelec G, Akbar A, Caruso C, Effros R, Grubeck-Loebenstein B, Wikby A. Is immunosenescence infectious? Trends Immunol **2004**; 25:406-10.

3. Ouyang Q, Wagner WM, Walter S, et al. An age-related increase in the number of CD8+ T cells carrying receptors for an immunodominant Epstein-Barr virus (EBV) epitope is counteracted by a decreased frequency of their antigen-specific responsiveness. Mech Ageing Dev **2003**; 124:477-85.

4. Darrah PA, Patel DT, De Luca PM, et al. Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat Med **2007**; 13:843-50.

5. Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol **2008**; 8:247-58.

6. Betts MR, Nason MC, West SM, et al. HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood **2006**; 107:4781-9.

7. Precopio ML, Betts MR, Parrino J, et al. Immunization with vaccinia virus induces polyfunctional and phenotypically distinctive CD8(+) T cell responses. J Exp Med **2007**; 204:1405-16.

8. Chiu YL, Lin CH, Sung BY, et al. Cytotoxic polyfunctionality maturation of cytomegalovirus-pp65-specific CD4 + and CD8 + T-cell responses in older adults positively correlates with response size. Sci Rep **2016**; 6:19227.

9. Lachmann R, Bajwa M, Vita S, et al. Polyfunctional T cells accumulate in large human cytomegalovirus-specific T cell responses. Journal of virology **2012**; 86:1001-9.

10. Sylwester A, Nambiar KZ, Caserta S, Klenerman P, Picker LJ, Kern F. A new perspective of the structural complexity of HCMV-specific T-cell responses. Mech Ageing Dev **2016**.

11. Sylwester AW, Mitchell BL, Edgar JB, et al. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J Exp Med **2005**; 202:673-85.

12. Terrazzini N, Bajwa M, Vita S, et al. A novel cytomegalovirus-induced regulatory-type T-cell subset increases in size during older life and links virus-specific immunity to vascular pathology. J Infect Dis **2014**; 209:1382-92.

13. Kern F, Faulhaber N, Frommel C, et al. Analysis of CD8 T cell reactivity to cytomegalovirus using protein-spanning pools of overlapping pentadecapeptides. Eur J Immunol **2000**; 30:1676-82.

14. Roederer M, Nozzi JL, Nason MC. SPICE: exploration and analysis of post-cytometric complex multivariate datasets. Cytometry A **2011**; 79:167-74.

15. Boyd A, Almeida JR, Darrah PA, et al. Pathogen-Specific T Cell Polyfunctionality Is a Correlate of T Cell Efficacy and Immune Protection. PLoS One **2015**; 10:e0128714.

16. Larsen M, Sauce D, Arnaud L, Fastenackels S, Appay V, Gorochov G. Evaluating cellular polyfunctionality with a novel polyfunctionality index. PLoS One **2012**; 7:e42403.

17. Lelic A, Verschoor CP, Ventresca M, et al. The polyfunctionality of human memory CD8+ T cells elicited by acute and chronic virus infections is not influenced by age. PLoS Pathog **2012**; 8:e1003076.

18. Van Epps P, Banks R, Aung H, Betts MR, Canaday DH. Age-related differences in polyfunctional T cell responses. Immun Ageing **2014**; 11:14.

19. Chiu YL, Shan L, Huang H, et al. Sprouty-2 regulates HIV-specific T cell polyfunctionality. J Clin Invest **2014**; 124:198-208.

20. Almeida JR, Sauce D, Price DA, et al. Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV-suppressive activity. Blood **2009**; 113:6351-60.

21. Kirchner A, Hoffmeister B, Cherepnev GG, et al. Dissection of the CMV specific T-cell response is required for optimized cardiac transplant monitoring. J Med Virol **2008**; 80:1604-14.

22. Ouyang Q, Wagner WM, Zheng W, Wikby A, Remarque EJ, Pawelec G. Dysfunctional CMV-specific CD8(+) T cells accumulate in the elderly. Exp Gerontol **2004**; 39:607-13.

23. Ouyang Q, Wagner WM, Wikby A, et al. Large numbers of dysfunctional CD8+ T lymphocytes bearing receptors for a single dominant CMV epitope in the very old. J Clin Immunol **2003**; 23:247-57.

24. Nieto FJ, Adam E, Sorlie P, et al. Cohort study of cytomegalovirus infection as a risk factor for carotid intimal-medial thickening, a measure of subclinical atherosclerosis. Circulation **1996**; 94:922-7.

25. Savva GM, Pachnio A, Kaul B, et al. Cytomegalovirus infection is associated with increased mortality in the older population. Aging cell **2013**; 12:381-7.

26. Spyridopoulos I, Martin-Ruiz C, Hilkens C, et al. CMV seropositivity and T-cell senescence predict increased cardiovascular mortality in octogenarians: results from the Newcastle 85+ study. Aging cell **2015**.

27. van de Berg PJ, Yong SL, Remmerswaal EB, van Lier RA, ten Berge IJ. Cytomegalovirus-induced effector T cells cause endothelial cell damage. Clin Vaccine Immunol **2012**; 19:772-9.

28. Solana R, Tarazona R, Aiello AE, et al. CMV and Immunosenescence: from basics to clinics. Immunity & ageing : I & A **2012**; 9:23.

29. Pawelec G, McElhaney JE, Aiello AE, Derhovanessian E. The impact of CMV infection on survival in older humans. Current opinion in immunology **2012**; 24:507-11.

30. Aiello AE, Simanek AM. Cytomegalovirus and immunological aging: the real driver of HIV and heart disease? The Journal of infectious diseases **2012**; 205:1772-4.

Figure Legends

Fig. 1: The functional T-cell response composition in young and older people appears very similar. PBMC from CMV+ donors were stimulated overnight with 19 CMV protein-derived overlapping peptide-pools. Activated T cells and their functional composition were analyzed by flow-cytometry, including the activation markers, degranulation (CD107), CD154, IL-2, TNF, and IFN-γ. Responses from the UK cohort are shown for the two most frequently targeted proteins for each T-cell subset. **(A)** Non-overlapping subsets exhibiting 5, 4, 3, or 2 parallel functions or just 1 single function are shown as a *proportion of the specifically activated* T-cells (order of decreasing polyfunctionality). A significant difference between the age groups is observed for UL-83 specific CD4 T-cells with 2 functions but on the whole subset distributions appear very similar (significance threshold set at $p \leq 0.01$, Mann-Whitney test, Bonferroni multiple endpoint correction, 5 end-points). **(B)** The *functional dominance* of each activation marker in a given response is assessed by the proportion of activated cells expressing it. Some variability between proteins is revealed but no significant differences between the age groups (significance threshold set at $p \leq 0.01$, Mann-Whitney test, Bonferroni multiple endpoint correction, 5 end-points).

Fig. 2: The polyfunctionality index of CMV-specific T-cell responses varies between protein targets and is reduced in the oldest old. The polyfunctionality index (PI) captures functional subset distributions by weighting the number of functions as well as subset size [15, 16]. For the present study a linear relationship between the number of functions and the relative weight of a subset was selected (e.g. subsets with

two functions were assigned twice the weight of subsets with one function, subsets with three functions were assigned three times the weight of subsets with one function, etc.). **(A)** In each individual the PI was calculated for all positive responses and averaged. Dot plots show the distribution of the average PI in young and older UK individuals for CD4 and CD8 T-cells. No significant differences between the groups were observed. **(B)** The comparison of the average PI between the older and the oldest old reveals significantly reduced polyfunctionality in the latter. **(C)** The PI of CD4 and CD8 T-cell responses is shown by target protein for the 5 most frequently recognized proteins in each T-cell compartment (each protein was recognized by 10 or more donors, only young and older UK donors were included in this analysis). Differences apparent for CD4 T-cells were not statistically significant (Kruskal-Wallis Test), but the data suggests that responses to UL83 were the most polyfunctional (upper left). However, significant differences are revealed for CD8 T-cells (upper right). Responses to UL83 showed the highest and responses to UL32 the lowest polyfunctionality. Lower panels show T-cell response size for each target protein (CD4 T-cells on the left, CD8 T-cells on the right), box plots show median, interquartile range, and outliers (o). **(D)** Interestingly, with respect to each analyzed protein, the PI significantly correlated with response size in a linear fashion. Correlations were moderate to strong. The strongest correlation was found for CD4 T-cell responses to UL83 (only UK donors were included in this analysis).

Tables

Table 1. CMV+ participant demographics (UK and Italy)

Parameter	**UK 'young'**	**UK 'older'**	**'oldest old'**
Total number	26	69	22
Age range (mean ± STD)	19 – 35 (23.3±4.2)	60 – 85 (69.0±7.5)	85 - 103 (95.9±5.9)
Females	18 (69 %)	35 (51 %)	16 (73%)
Males	8 (31 %)	34 (49 %)	6 (27%)
White (British or Italian)	18 (69%)	69 (100 %)	22 (100%)
Non-White British[a]	8 (31%)	0 (0%)	0 (0%)

[a]Non-white British young adults included 1 Syrian, 2 Indian, 1 Sri-Lankan, 1 Bangladeshi, 1 Malaysian, 1 White/Asian and 1 Black African/Asian participants.

Table 2: CMV peptide-pools used for stimulation

Tube	Protein(s)	No. of Peptides
1	UL55[5]	224
2	UL83	138
3	UL86	340
4	UL122	120
5	UL123	143
6	UL99	45
7	UL153	67
8	UL32	260
9	UL28	92
10	UL48A[a]	281
11	UL48B[a]	281
12	US3	44
13	UL151& UL82	219 (82+137)
14	UL94 & US29	197 (84+113)
15	UL103 & US32	103 (60+43)
16	US24 & UL36	240 (123+117)

[a] UL48 was divided into two pools for stimulation (UL48A and UL48B),however, results were combined

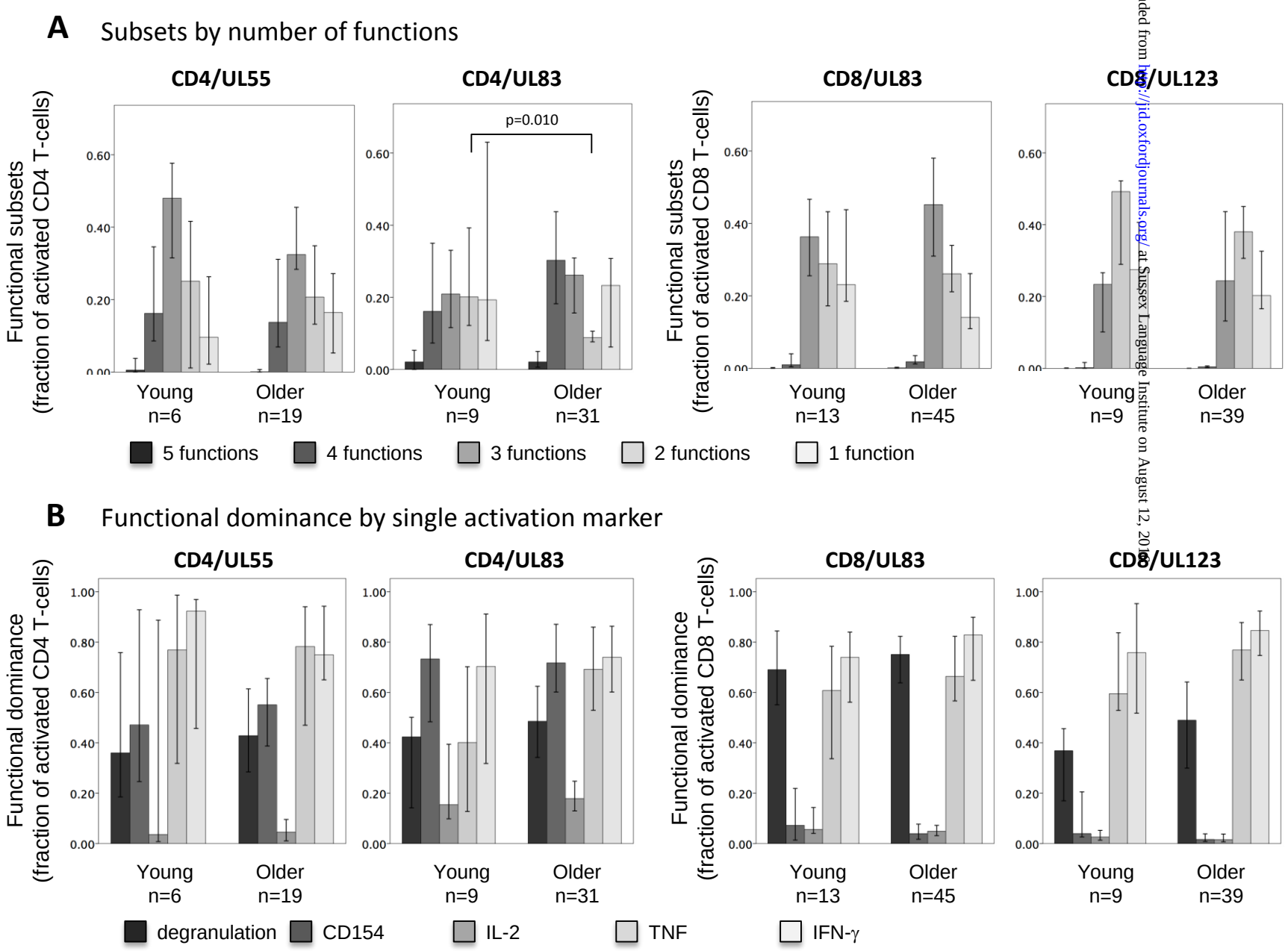

Figure 1

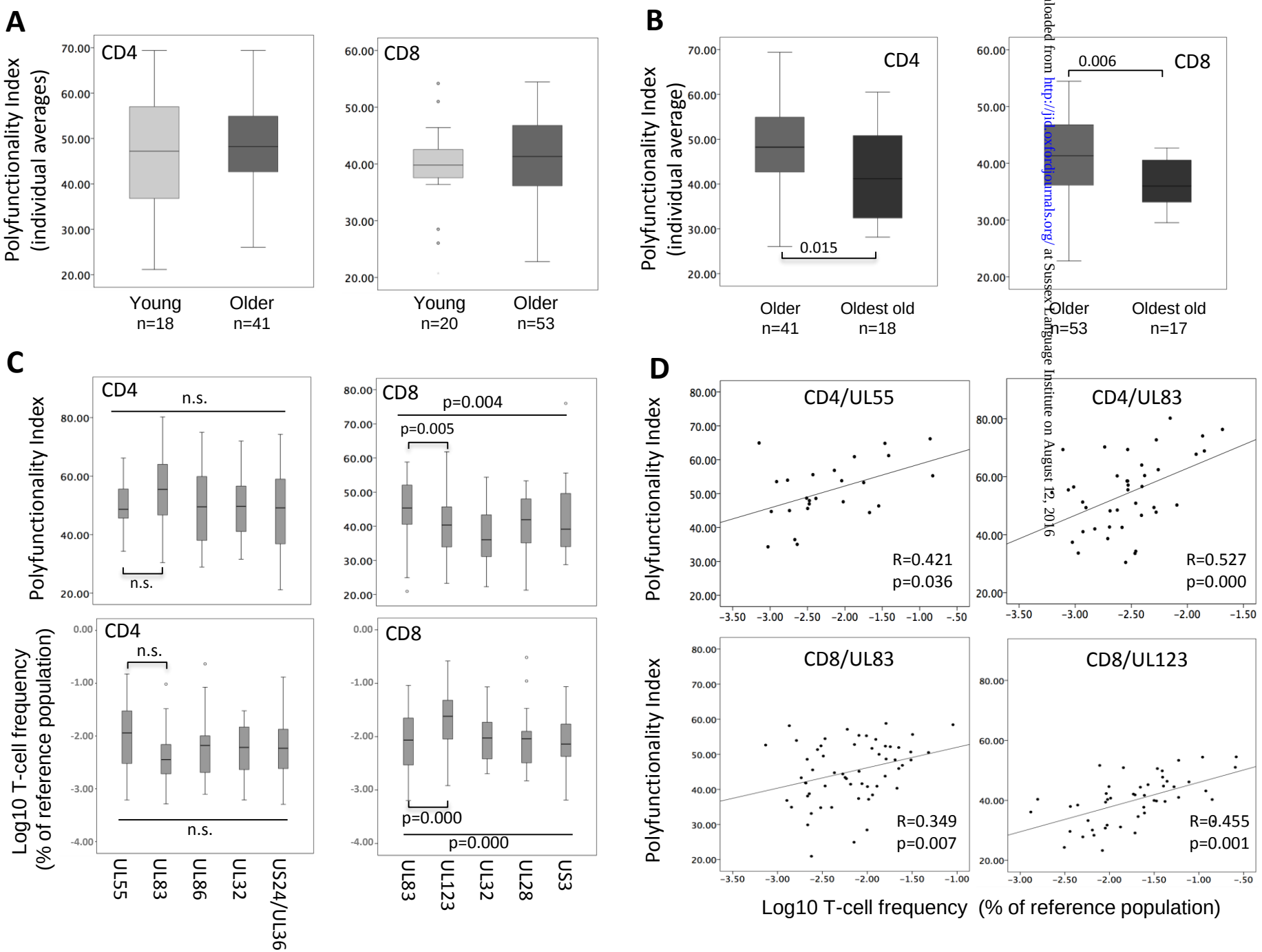

Figure 2